CLASSIFYING CANCERS

Reference to Material Presented in Appendix

This patent application includes material comprising tables and data presented as Appendix A on CD-ROM. The one file on the accompanying CD-ROM is entitled AppendixA.xls (2,868 kb), which is a Microsoft Excel Worksheet. The CD-ROM was created on August 2, 2001. The format is IBM-PC. The operating system is MS-Windows 98. The file on the CD-ROM is incorporated herein by reference.

Background of the Invention

Cancer is the second leading cause of death in the United States after cardiovascular disease (Boring *et al. Cancer J. Clin.* 43:7, 1993; incorporated herein by reference). One in three Americans will develop cancer in his or her lifetime, and one of every four Americans will die of cancer. In order to better combat this deadly disease, efforts have recently focused on fine tuning the categorization of tumors; by categorizing cancers, physicians hope to better treat an individual's cancer by providing more effective treatments. Researchers and physicians have categorized cancers based on invasion, metastasis, gross pathology, microscopic pathology, imunohistochemical markers, and molecular markers. With the recent advances in gene chip technology, researchers are increasingly focusing on the categorization of tumors based on the expression of marker genes.

The most common human cancers are malignant neoplasms of the skin (Hall *et al. J. Am. Acad. Dermatol.* 40:35-42, 1999; Weyers *et al. Cancer* 86:288-299, 1999; each of which is incorporated herein by reference). The incidence of cutaneous melanoma is rising especially steeply, with minimal progress in non-surgical treatment of advanced disease (Byers *et al. Hematol. Oncol. Clin. North Am.* 12:717-735, 1998; McMasters *et al. Ann. Surg.*

Oncol. 6:467-475, 1999; each of which is incorporated herein by reference). Despite significant effort to identify independent predictors of melanoma outcome, no accepted histopathological, molecular, or immunohistochemical marker defines subsets of this neoplasm (Weyers *et al. Cancer* 86:288-299, 1999; Byers *et al. Hematol. Oncol. Clin. North Am.* 12:717-735, 1998; each of which is incorporated herein by reference). Accordingly, though melanoma is thought to present with different "taxonomic" forms, these are considered part of a continuous spectrum rather than discrete entities (Weyers *et al. Cancer* 86:288-299, 1999; incorporated herein by reference). Improved characterization and understanding of this potentially deadly disease would be valuable.

Summary of the Invention

The present invention provides a system for diagnosing aggressive forms of malignant melanoma based on the expression of certain marker genes within a tumor sample. In one embodiment, expression levels are determined for one or more of the following genes: Wnt5a (Seq. ID No.: 1, 2, & 3), MART-1 (Seq. ID No.: 4 & 5), pirin (Seq. ID No.: 6 & 7), HADHB (Seq. ID No.: 8 & 9), CD63 (Seq. ID No.: 10 & 11), EDNRB (Seq. ID No.: 12 & 13), PGAM1 (Seq. ID No.: 14 & 15), HXB (Seq. ID No.: 16 & 17), RXRA (Seq. ID No.: 18 & 19), integrin 1b (Seq. ID No.: 20 & 21), syndecan 4 (Seq. ID No.: 22 & 23), tropomyosin 1 (Seq. ID No.: 24 & 25), AXL (Seq. ID No.: 26 & 27), EphA2 (Seq. ID No.: 28 & 29), GAP43 (Seq. ID. No.: 30 & 31), PFKL (Seq. ID No.: 32 & 33), synuclein a (Seq. ID No.: 34 & 35), annexin A2 (Seq. ID No.: 36 & 37), CD20 (Seq. ID No.: 38 & 39), and RAB2 (Seq. ID No.: 40 & 41). In certain preferred embodiments, expression of a plurality of these genes is detected. In particularly preferred embodiments, *Wnt5a* is one of the genes

whose expression is detected. According to the present invention, overexpression of *Wnt5a* in a tumor sample indicates a more aggressive form of the disease.

The present invention also provides a system for selecting a treatment protocol for a patient diagnosed with malignant melanoma based on the expression pattern of certain marker genes in a tumor sample. For example, tumors overexpressing *Wnt5a* may be treated more aggressively or with specific agents such as inhibitors of *Wnt5a* expression. Inhibitors of *Wnt5a* activity include anti-sense agents, RNA inhibition agents, small molecule inhibitors of *Wnt5a* activity, gene therapy, *etc.*

In another aspect, the present invention provides a system for identifying and then treating aggressive forms of malignant melanoma by administering inhibitors of *Wnt5a* activity to a subject.

In another aspect, the present invention provides a system for identifying compounds useful in the treatment of cancer, particularly aggressive forms of malignant melanoma expressing *Wnt5a*. In the inventive method, a cell expressing *Wnt5a* is contacted with an agent being screened for activities useful in the treatment of cancer, such as decreasing or inhibiting *Wnt5a* expression and/or activity. The agent may be a polynucleotide, protein, peptide, natural product, small molecule, *etc.* The level of *Wnt5a* expression or activity may be assayed using any available technique, including but not limited to, Northern blot analysis, enzyme activity, expression of a reporter gene, *etc.*

The present invention also provides kits useful in diagnosing or identifying cancers or more aggressive forms of cancer. The kits may be used to identify more aggressive forms of malignant melanoma. The kit may include a gene chip with nucleic acid sequences of genes of interest including Wnt5a, MART-1, pirin, HADHB, CD63, EDNRB, PGAM1, HXB, RXRA, integrin 1b, syndecan 4, tropomyosin 1, AXL, EphA2, GAP43, PFKL, synuclein a,

annexin A2, CD20, and RAB2, or a subset thereof. The kit may also or alternatively include primers, enzymes, and reagents for identifying, amplifying, labeling, or sequencing nucleic acids. Same kits may also include reagents for purifying nucleic acids such as mRNA. Rather than detecting gene expression, the kit may be used to determine protein levels and therefore include antibodies directed against the proteins encoded by the genes, Wnt5a, MART-1, pirin, HADHB, CD63, EDNRB, PGAM1, HXB, RXRA, integrin 1b, syndecan 4, tropomyosin 1, AXL, EphA2, GAP43, PFKL, synuclein a, annexin A2, CD20, and RAB2, or a subset thereof.

Definitions

"Animal": The term animal, as used herein, refers to humans as well as non-human animals, including, for example, mammals, birds, reptiles, amphibians, and fish. Preferred non-human animals are a mammals (*e.g.*, a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a primate, or a pig). An animal may be a transgenic animal. In certain embodiments, non-human animals may be laboratory animals, raised by humans in a controlled environment other than their natural habitat.

"Antibody": The term antibody refers to an immunoglobulin, whether natural or wholly or partially synthetically produced. All derivatives thereof which maintain specific binding ability are also included in the term. The term also covers any protein having a binding domain which is homologous or largely homologous to an immunoglobulin binding domain. These proteins may be derived from natural sources, or partly or wholly synthetically produced. An antibody may be monoclonal or polyclonal. The antibody may be a member of any immunoglobulin class, including any of the human classes: IgG, IgM,

IgA, IgD, and IgE. The antibody may be a fragment of an antibody such as an Fab fragment or a recombinantly produced scFv fragment.

"Cancer": Cancer refers to a malignant tumor (*e.g.*, lung cancer) or growth of cells (*e.g.*, leukemia). Cancers tend to be less differentiated than benign tumors, grow more rapidly, show infiltration, invasion and destruction, and may metastasize. Cancers include, but are not limited to, fibrosarcoma, myxosarcoma, angiosarcoma, leukemia, squamous cell carcinoma, basal cell carcinoma, malignant melanoma, renal cell carcinoma, hepatocellular carcinoma, *etc.*

"Effective amount": In general, the "effective amount" of an active agent refers to the amount necessary to elicit a desired biological response. As will be appreciated by those of ordinary skill in this art, the absolute amount of a Wnt5a inhibitor that is effective may vary depending on such factors as the desired biological endpoint, the agent to be delivered, the target tissue, *etc.* Those of ordinary skill in the art will further understand that an "effective amount" may be administered in a single dose, or may be achieved by administration of multiple doses. For example, in the case of anti-neoplastic agents, the effective amount may be the amount of agent needed to reduce the size of the primary tumor, to reduce the size of a secondary tumor, to reduce the number of metastases, to reduce the growth rate of a tumor, to reduce the ability of the primary tumor to metastasize, to increase life expectancy, *etc.*.

"Marker gene": A "marker gene" may be any gene or gene product (*e.g.*, protein, peptide, mRNA) that indicates a particular diseased or physiological state (*e.g.*, carcinoma, normal, dysplasia) or indicates a particular cell type, tissue type, or origin. The expression or lack of expression of a marker gene may indicate a particular physiological or diseased state of a patient, organ, tissue, or cell. Preferably, the expression or lack of expression may be determined using standard techniques such as RT-PCR, sequencing, immunochemistry, gene

chip analysis, *etc.* In certain embodiments, the level of expression of a marker gene is quantifiable.

"Peptide" or "protein": According to the present invention, a "peptide" or "protein" comprises a string of at least three amino acids linked together by peptide bonds. The terms "protein" and "peptide" may be used interchangeably. Peptide may refer to an individual peptide or a collection of peptides. Inventive peptides preferably contain only natural amino acids, although non-natural amino acids (*i.e.*, compounds that do not occur in nature but that can be incorporated into a polypeptide chain; see, for example, http://www.cco.caltech.edu/~dadgrp/Unnatstruct.gif, which displays structures of non-natural amino acids that have been successfully incorporated into functional ion channels) and/or amino acid analogs as are known in the art may alternatively be employed. Also, one or more of the amino acids in an inventive peptide may be modified, for example, by the addition of a chemical entity such as a carbohydrate group, a phosphate group, a farnesyl group, an isofarnesyl group, a fatty acid group, a linker for conjugation, functionalization, or other modification, *etc.* In a preferred embodiment, the modifications of the peptide lead to a more stable peptide (*e.g.*, greater half-life *in vivo*). These modifications may include cyclization of the peptide, the incorporation of D-amino acids, *etc.* None of the modifications should substantially interfere with the desired biological activity of the peptide.

"Polynucleotide" or "oligonucleotide": Polynucleotide or oligonucleotide refers to a polymer of nucleotides. Typically, a polynucleotide comprises at least three nucleotides. The polymer may include natural nucleosides (*i.e.*, adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine), nucleoside analogs (*e.g.*, 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, C5-propynylcytidine, C5-propynyluridine, C5-bromouridine,

C5-fluorouridine, C5-iodouridine, C5-methylcytidine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, and 2-thiocytidine), chemically modified bases, biologically modified bases (*e.g.*, methylated bases), intercalated bases, modified sugars (*e.g.*, 2′-fluororibose, ribose, 2′-deoxyribose, arabinose, and hexose), or modified phosphate groups (*e.g.*, phosphorothioates and 5′-N-phosphoramidite linkages).

"Small molecule": As used herein, the term "small molecule" refers to organic compounds, whether naturally-occurring or artificially created (*e.g.*, via chemical synthesis) that have relatively low molecular weight and that are not proteins, polypeptides, or nucleic acids. Typically, small molecules have a molecular weight of less than about 1500 g/mol. Also, small molecules typically have multiple carbon-carbon bonds.

"Tumor": As used in the present application, "tumor" refers to an abnormal growth of cells. The growth of the cells of a tumor typically exceed the growth of normal tissue and tends to be uncoordinated. The tumor may be benign (*e.g.*, lipoma, fibroma, myxoma, lymphangioma, meningioma, nevus, adenoma, leiomyoma, mature teratoma, *etc.*) or malignant (*e.g.*, malignant melanoma, ovarian cancer, carcinoma *in situ*, carcinoma, adenocarcinoma, liposarcoma, mesothelioma, squamous cell carcinoma, basal cell carcinoma, colon cancer, lung cancer, *etc.*).

Brief Description of the Drawing

Figure 1 shows the clustering of gene expression data. **a**. Hierarchical clustering dendrogram with the cluster of 19 melanomas at the center. **b**. MDS three-dimensional plot of all 31 cutaneous melanoma samples showing major clustering of 19 samples (blue, within cylinder), and remaining 12 samples (gold). **c**. A plot of the observed and expected number

of genes producing a given number of classification errors for a partition of the 31 melanomas into two groups of 12 and 19. Red triangles, observed clusters; filled bars, randomly produced clusters, open circles, predicted results for randomly variable gene expression. **d**. Introduction of random gaussian noise followed by cuts from the top of the original tree (resulting in *k* clusters), to determine discrepant pairs after perturbation (see Supplementary Information in Examples).

Figure 2 illustrates the identification of genes which discriminate melanoma clusters. **a**. MDS analysis ranking genes according to their impact on minimizing cluster volume and maximizing center-to center inter-cluster distance. **b**. Top 22 genes obtained by these criteria listed in order of decreasing weight (for a full list, see Supplementary Information in Examples). Right, data from cutaneous melanomas identified on the horizontal axis and sorted by cluster (described in Maniotis *et al.* "Vascular channel formation by human melanoma cells *in vivo* and *in vitro*: vasculogenic mimicry" *Am. J. Pathol.* 155:739-752, 1999; incorporated herein by reference). Left, data from uveal melanomas expressed as the ratio of highly invasive to less invasive. Red, high ratios; green, low ratios (intensity of saturation scaled according to the ratio). The three genes not scored in the uveal samples were not included in the print design of the cutaneous samples.

Figure 3. Guiding gene cluster selection. **a**. Two-dimensional cluster analysis of cutaneous melanoma samples (horizontal axis) and genes (vertical axis, presented in segments). **b-e**. Data from a queried at regions corresponding to four two discriminators of the major cluster: MART-1 (**b**), CD63 (**c**), tropomyosin (**d**), and WNT5a (**e**). Note that these clusters include other genes from the discriminator list (bold). The major cluster of 19 samples is visually apparent on the left of this display. The full list of gene names and

corresponding calculated ratio information is provided in the Supplementary Information in the Examples.

Figure 4 shows the variation in biological properties of melanoma clusters. **a-c**. A representative member of the major melanoma cluster (UACC-1022). **d-f**. A sample falling outside of the major cluster (M93-047). The two groups differ in the ability to migrate into a scratch wound (**a**, **d**), contract collagen gels (**b**, **e**) and form tubular networks (**c**, **f**). Results of these and additional cell mobility/invasion assays are included in Table 1. Tubular network formation (vasculogenic mimicry (Maniotis *et al.* "Vascular channel formation by human melanoma cells *in vivo* and *in vitro*: vasculogenic mimicry" *Am. J. Pathol.* 155:739-752, 1999; incorporated herein by reference), **f**) and collagen gel contraction (related to the patterning of vascular channels, **e**) were observed only outside the major cluster (Table 1).

Figure 5 shows a Kaplan-Meier survival plot for a total of 15 cases, 10 from Group A and 5 from Group B. No statistically significant association between group and survival was found ($p = 0.135$).

Figure 6 shows the data obtained from the top 22 genes with *Wnt5a* at the top of the list. The figure also show a diagram of the *Wnt5a* and *Wnt1* signaling pathways.

Figure 7 shows the data from real time PCR analysis of three cell lines, one with low *Wnt5a* expression (which scored as having low expression in the gene chip analysis), one with high *Wnt5a* expression (which scored as having high expression in the gene chip analysis), and one with intermediate *Wnt5a* expression, an originally low scoring cell line which had been transfected with a vector designed to express *Wnt5a*. The patent and transfected cell line were also analyzed for WNT5A protein abundance using Western blot analysis and immunohistochemical staining.

Figure 8 shows the dramatic changes in cell morphology and cytoskeletal organization upon transfection of the parental cell line with a vector driving *Wnt5a* expression. The parental cell line is spindle shaped with few points of attachment to the culture plate and disorganized actin filaments. The transfectants are broader and flatter with many extensions and highly polarlized actin filaments.

Figure 9 shows the results of experiments done to look at possible cross talk between the *Wnt5a* and *Wnt1* pathways. Beta-catenin was localized to the cytoplasm indicating that the *Wnt1* pathway is not active. The downstream target of *Wnt5a*, protein kinase C, was also observed to be phosphorylated, especially the mu and alpha/beta isoforms, indicating that the expected *Wnt5a* pathway is active.

Figure 10 shows scratch assay and Boyden chamber assay results for the parent cell line as well as the transfected cell line. The results from these two standard assays show that increased cell movement and invasiveness correlate with increased *Wnt5a* expression.

Figure 11 shows that the transition from low to high *Wnt5a* expression is not associated with increasing amounts of the G protein coupled receptor, frizzled 5 (fzd5). Also shown are results indicating that an antibody to fzd5 can attenuate or reverse the phenotype that increased *Wnt5a* would normally produce.

Detailed Description of Certain Preferred Embodiments of the Invention

The present invention provides systems for identifying and treating cancers based on the expression of marker genes in the cancer cells. In a particular embodiment, the cancer to be categorized is malignant melanoma. The invention allows for the identification of more aggressive forms of cancer and profiling the affected patient so that a proper treatment

regimen can be initiated. The present invention also provides for kits useful in practicing the inventive methods.

Diagnosing and Identifying Forms of Cancer

In diagnosing or identifying a particular cancer or tumor, a test sample containing at least one cell from the tumor is provided to obtain a genetic sample. The test sample may be obtained using any technique known in the art including biopsy, blood sample, sample of bodily fluid (*e.g.*, urine, lymph, ascites, cerebral spinal fluid, pleural effusion, sputum, stool, tears, sweat, pus, *etc.*), surgical excisions. needle biopsy, scraping, *etc.* From the test sample is obtained a genetic sample. The genetic sample comprises a nucleic acid, preferably RNA and/or DNA. For example, in determining the expression of marker genes one can obtain mRNA from the test sample, and the mRNA may be reverse transcribed into cDNA for further analysis. In another embodiment, the mRNA itself is used in determining the expression of marker genes. In some embodiments, the expressions level of a particular marker gene may be determined by determining the level/presence of a gene product (*e.g.*, protein) thereby eliminating the need to obtain a genetic sample from the test sample.

The test sample is preferably a sample representative of the tumor or cancer as a whole. Preferably there is enough of the test sample to obtain a large enough genetic sample to accurately and reliably determine the expression levels of marker genes of interest in the cancer or tumor. In certain embodiments, multiple samples may be taken from the same tumor in order to obtain a representative sampling of the tumor.

A genetic sample may be obtained from the test sample using any techniques known in the art (Ausubel *et al. Current Protocols in Molecular Biology* (John Wiley & Sons, Inc., New York, 1999); *Molecular Cloning: A Laboratory Manual*, 2nd Ed., ed. by Sambrook,

Fritsch, and Maniatis (Cold Spring Harbor Laboratory Press: 1989); *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); the treatise, *Methods in Enzymology* (Academic Press, Inc., N.Y.); each of which is incorporated herein by reference). The nucleic acid may be purified from whole cells using DNA or RNA purification techniques. The genetic sample may also be amplified using PCR or *in vivo* techniques requiring subcloning. In a preferred embodiment, the genetic sample is obtained by isolating mRNA from the cells of the test sample and reverse transcribing the RNA into DNA in order to create cDNA (Khan *et al. Biochem. Biophys. Acta* 1423:17-28, 1999; incorporated herein by reference).

Once a genetic sample has been obtained, it can be analyzed for the presence or absence of particular marker genes. The analysis may be performed using any techniques known in the art including, but not limited to, sequencing, PCR, RT-PCR, quantitative PCR, restriction fragment length polymorphism, hybridization techniques, Northern blot, microarray technology, DNA microarray technology, *etc.* In determining the expression level of a marker gene or genes in a genetic sample, the level of expression may be normalized by comparison to the expression of another gene such as a well known, well characterized gene or a housekeeping gene.

The expression data from a particular marker gene or group of marker genes may be analyzed using statistical methods described below in the Examples in order to determine the phenotype or characteristic of a particular tumor or cancer. Methods used in classifying tumors based on gene expression data are described in Ben-Dor *et al. J. Comput. Biol.* 7(3 & 4):559-584, 2000; incorporated herein by reference. The analyzed data may also be used to select/profile patients for a particular treatment protocol.

For example, the present invention demonstrates that marker gene *Wnt5a* is expressed at high levels in more aggressive forms of malignant melanomas. A patient with malignant melanoma may have the expression level of *Wnt5a* in the cells of his/her tumor determined in order to help determine the prognosis and/or treatment plan for his/her particular disease. The expression level of *Wnt5a* would preferably be one of several factors used in deciding the prognosis or treatment plan of a patient. Preferably a trained and fully licensed physician would be consulted in determining the patient's prognosis and treatment plan. A high level of expression of *Wnt5a* may indicate a worse prognosis and suggest a more aggressive treatment plan. The treatment plan may also include inhibitors of *Wnt5a* activity such as anti-sense agents and gene therapy directed against *Wnt5a*. Small molecule inhibitors of *Wnt5a* activity may also be used in the treatment plan as well as pharmaceuticals that inhibit the *Wnt5a* pathway either upstream or downstream of *Wnt5a* itself.

Marker Genes

The present invention provides several marker genes that correlate with particularly aggressive forms of malignant melanoma. These markers may also be useful in categorizing other tumors or cancers other than malignant melanoma. For example, inventive marker genes may be useful in categorizing other types of skin cancer. Preferred marker genes include Wnt5a, MART-1, pirin, HADHB, CD63, ENDRB, PGAM1, HXB, RXRA, integrin b1, syndecan 4, tropomyosin 1, AXL, EphA2, GAP43, PFKL, synuclein a, annexin A2, CD20, and RAB2, and combinations thereof. Other potential marker genes are listed in the Examples below. Particular sets of marker genes may be defined using statistical methods as described in the Examples in order to decrease or increase the specificity or sensitivity of the set. For example, a particular set of marker genes highly specific of aggressive forms of

malignant melanoma may be less sensitive (*i.e.*, a negative result may occur in the presence on an aggressive form of melanoma).

Different subsets of marker genes may be developed that show optimal function with different races, ethnic groups, sexes, geographic groups, stages of disease, types of cancer, cell types, *etc.* Subsets of marker genes may also be developed to be sensitive to the effect of a particular therapeutic regimen on disease progression.

One particularly useful marker gene in the diagnosis of aggressive form of malignant melanoma is *Wnt5a*. The *Wnt* genes make up a large family of highly conserved genes that have been studied extensively in development. The first member, *int-1* was discovered as a common integration site of mouse mammary tumor virus (MMTV) in mammary epithelial adenocarcinomas (Nusse and Varmus *Cell* 69:1073-1087, 1992; incorporated herein by reference). *Int-1* is highly homologous to the *Drosophila* developmental gene *wingless* that is involved in pattern formation. The combination of *wingless* and *int-1* gives rise to the term *Wnt*. Homologues of *Wnt* genes have been isolated in *Drosophila*, *Xenopus*, chicken, mouse, and humans (Nusse and Varmus *Cell* 69:1073-1087, 1992; incorporated herein by reference). In humans, there are nine *Wnt* genes known including *Wnt5a* (Clark *et al. Genomics* 18:249-260, 1993; Lejeune *et al. Clin. Cancer Res.* 1:215-222, 1995; each of which is incorporated herein by reference). *Wnt5a* has been found to be up-regulated in lung, colon, and prostate carcinomas and melanomas (Iozzo *et al. Cancer Res.* 55:3495-3499, 1995; incorporated herein by reference).

The sequence of the mRNA of *Homo sapiens* wingless MMTV integration site family, member 5a (*Wnt5a*) is shown below:

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        1 attaattctg gctccacttg ttgctcggcc caggttgggg agaggacgga
gggtggccgc
       61 agcgggttcc tgagtgaatt acccaggagg gactgagcac agcaccaact
agagaggggt
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121 caggggtgc gggactcgag cgagcaggaa ggaggcagcg cctggcacca gggctttgac

181 tcaacagaat tgagacacgt ttgtaatcgc tggcgtgccc cgcgcacagg atcccagcga

241 aaatcagatt tcctggtgag gttgcgtggg tggattaatt tggaaaaaga aactgcctat

301 atcttgccat caaaaaactc acggaggaga agcgcagtca atcaacagta aacttaagag

361 acccccgatg ctcccctggt ttaacttgta tgcttgaaaa ttatctgaga gggaataaac

421 atcttttcct tcttccctct ccagaagtcc attggaatat taagcccagg agttgctttg

481 gggatggctg gaagtgcaat gtcttccaag ttcttcctag tggctttggc catatttttc

541 tccttcgccc aggttgtaat tgaagccaat tcttggtggt cgctaggtat gaataaccct

601 gttcagatgt cagaagtata tattatagga gcacagcctc tctgcagcca actggcagga

661 ctttctcaag gacagaagaa actgtgccac ttgtatcagg accacatgca gtacatcgga

721 gaaggcgcga agacaggcat caaagaatgc cagtatcaat tccgacatcg acggtggaac

781 tgcagcactg tggataacac ctctgttttt ggcagggtga tgcagatagg cagccgcgag

841 acggccttca catacgccgt gagcgcagca ggggtggtga acgccatgag ccgggcgtgc

901 cgcgagggcg agctgtccac ctgcggctgc agccgcgccg cgcgccccaa ggacctgccg

961 cgggactggc tctggggcgg ctgcggcgac aacatcgact atggctaccg ctttgccaag

1021 gagttcgtgg acgcccgcga gcgggagcgc atccacgcca agggctccta cgagagtgct

1081 cgcatcctca tgaacctgca caacaacgag gccggccgca ggacggtgta caacctggct

1141 gatgtggcct gcaagtgcca tggggtgtcc ggctcatgta gcctgaagac atgctggctg

1201 cagctggcag acttccgcaa ggtgggtgat gccctgaagg agaagtacga cagcgcggcg

1261 gccatgcggc tcaacagccg gggcaagttg gtacaggtca acagccgctt caactcgccc

1321 accacacaag acctggtcta catcgacccc agccctgact actgcgtgcg caatgagagc

1381 accggctcgc tgggcacgca gggccgcctg tgcaacaaga cgtcggaggg catggatggc

1441 tgcgagctca tgtgctgcgg ccgtgggtac gaccagttca agaccgtgca gacggagcgc

1501 tgccactgca agttccactg gtgctgctac gtcaagtgca agaagtgcac ggagatcgtg

1561 gaccagtttg tgtgcaagta gtgggtgcca cccagcactc agccccgctc ccaggacccg

1621 cttatttata gaaagtacag tgattctggt ttttggtttt tagaaatatt ttttatttttt

1681 ccccaagaat tgcaaccgga accatttttt ttcctgttac catctaagaa ctctgtggtt

1741 tattattaat attataatta ttatttggca ataatggggg tgggaaccac gaaaaatatt
1801 tattttgtgg atctttgaaa aggtaataca agacttcttt tggatagtat agaatgaagg
1861 gggaaataac acatacccta acttagctgt gtgggacatg gtacacatcc agaaggtaaa
1921 gaaatacatt ttctttttct caaatatgcc atcatatggg atgggtaggt tccagttgaa
1981 agagggtggt agaaatctat tcacaattca gcttctatga ccaaaatgag ttgtaaattc
2041 tctggtgcaa gataaaaggt cttgggaaaa caaaacaaaa caaaacaaac ctcccttccc
2101 cagcagggct gctagcttgc tttctgcatt ttcaaaatga taatttacaa tggaaggaca
2161 agaatgtcat attctcaagg aaaaaaggta tatcacatgt ctcattctcc tcaaatattc
2221 catttgcaga cagaccgtca tattctaata gctcatgaaa tttgggcagc agggaggaaa
2281 gtccccagaa attaaaaaat ttaaaactct tatgtcaaga tgttgatttg aagctgttat
2341 aagaattggg attccagatt tgtaaaaaga cccccaatga ttctggacac tagatttttt
2401 gtttggggag gttggcttga acataaatga aatatcctgt attttcttag ggatacttgg
2461 ttagtaaatt ataatagtag aaataataca tgaatcccat tcacaggttt ctcagcccaa
2521 gcaacaaggt aattgcgtgc cattcagcac tgcaccagag cagacaacct atttgaggaa
2581 aaacagtgaa atccaccttc ctcttcacac tgagccctct ctgattcctc cgtgttgtga
2641 tgtgatgctg gccacgtttc caaacggcag ctccactggg tccccttttgg ttgtaggaca
2701 ggaaatgaaa cattaggagc tctgcttgga aaacagttca ctacttaggg atttttgttt
2761 cctaaaactt ttattttgag gagcagtagt tttctatgtt ttaatgacag aacttggcta
2821 atggaattca cagaggtgtt gcagcgtatc actgttatga tcctgtgttt agattatcca
2881 ctcatgcttc tcctattgta ctgcaggtgt accttaaaac tgttcccagt gtacttgaac
2941 agttgcattt ataaggggggg aaatgtggtt taatggtgcc tgatatctca aagtcttttg
3001 tacataacat atatatatat atacatatat ataaatataa atataaatat atctcattgc
3061 agccagtgat ttagatttac agcttactct ggggttatct ctctgtctag agcattgttg
3121 tccttcactg cagtccagtt gggattattc caaaagtttt ttgagtcttg agcttgggct
3181 gtggccccgc tgtgatcata ccctgagcac gacgaagcaa cctcgtttct gaggaagaag
3241 cttgagttct gactcactga aatgcgtgtt gggttgaaga tatctttttt tcttttctgc
3301 ctcacccctt tgtctccaac ctccatttct gttcactttg tggagagggc attacttgtt

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     3361 cgttatagac atggacgtta agagatattc aaaactcaga agcatcagca
atgtttctct
     3421 tttcttagtt cattctgcag aatggaaacc catgcctatt agaaatgaca
gtacttatta
     3481 attgagtccc taaggaatat tcagcccact acatagatag ctttttttt
ttttttttt
     3541 ttttaataag gacacctctt tccaaacagg ccatcaaata tgttcttatc
tcagacttac
     3601 gttgttttaa aagtttggaa agatacacat cttttcatac ccccccttag
gaggttgggc
     3661 tttcatatca cctcagccaa ctgtggctct taatttattg cataatgata
tccacatcag
     3721 ccaactgtgg ctctttaatt tattgcataa tgatattcac atcccctcag
ttgcagtgaa
     3781 ttgtgagcaa aagatcttga aagcaaaaag cactaattag tttaaaatgt
cacttttttg
     3841 gtttttatta tacaaaaacc atgaagtact ttttttattt gctaaatcag
attgttcctt
     3901 tttagtgact catgtttatg aagagagttg agtttaacaa tcctagcttt
taaaagaaac
     3961 tatttaatgt aaaatattct acatgtcatt cagatattat gtatatcttc
tagcctttat
     4021 tctgtacttt taatgtacat atttctgtct tgcgtgattt gtatatttca
ctggtttaaa
     4081 aaacaaacat cgaaaggctt attccaaatg gaag
```

The translated sequence of *Wnt5a* is as follows:

MAGSAMSSKFFLVALAIFFSFAQVVIEANSWWSLGMNNPVQMSE
VYIIGAQPLCSQLAGLSQGQKKLCHLYQDHMQYIGEGAKTGIKECQYQFRHRRWNCST
VDNTSVFGRVMQIGSRETAFTYAVSAAGVVNAMSRACREGELSTCGCSRAARPKDLPR
DWLWGGCGDNIDYGYRFAKEFVDARERERIHAKGSYESARILMNLHNNEAGRRTVYNL
ADVACKCHGVSGSCSLKTCWLQLADFRKVGDALKEKYDSAAAMRLNSRGKLVQVNSRF
NSPTTQDLVYIDPSPDYCVRNESTGSLGTQGRLCNKTSEGMDGCELMCCGRGYDQFKT
VQTERCHCKFHWCCYVKCKKCTEIVDQFVCK (Seq. ID No.: 3)

Other sequences homologous to the above sequences may also be used in the present invention. Preferably the sequence is at least 70% identical to the human *Wnt5a* DNA and protein sequences listed above. More preferably the sequence is at least 80%, 90%, 95%, 97%, 98%, 99%, or >99% identical. A homolog of *Wnt5a* may also be identified by its activity. In another preferred embodiment, the homolog of *Wnt5a* is identified by its location in the genome (*e.g.*, location on the chromosome).

Identifying Anti-Neoplastic Agents

The present invention also provides a novel method of identifying compounds useful in the treatment of patients with cancer. In certain embodiments, the cancer is malignant melanoma. In other embodiments, the cancer is a malignant melanoma expressing *Wnt5a*. In particular, the inventive method identifies compounds directed against *Wnt5a* or *Wnt5a* activity specifically, or more generally, against downstream or upstream signals in the *Wnt5a* pathway.

Any compound, moiety, or entity can be screened for activity against *Wnt5a* according to the present invention. For example, polynucleotides, peptides, proteins, natural products, chemical compounds, small molecules, polymers, biomolecules, *etc.* may be tested. The agents to be screened may be prepared by purification or synthesis, or may be obtained from commercial or other stock sources.

The assay used to screen the agents may be an *in vitro* or *in vivo* assay. For example, an *in vitro* assay may utilize purified or partially purified WNT5A protein. The WNT5A protein may be obtained by purifying the protein from a natural source or from a cell, such as bacteria, mammalian cells, yeast, or fungi, overexpressing WNT5A. Methods for overexpressing and purifying the proteins encoded by cloned genes are well known in the art (see, Ausubel *et al. Current Protocols in Molecular Biology* (John Wiley & Sons, Inc., New York, 1999); *Molecular Cloning: A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch, and Maniatis (Cold Spring Harbor Laboratory Press: 1989; each of which is incorporated herein by reference). Agents may be screened for their ability to bind the WNT5A protein or to enhance or prevent an interaction between WNT5A and another protein, peptide, polynucleotide, or chemical compound. Agents may also be screened for their ability to affect more downstream effects of WNT5A. Agents may be screened using high-throughput techniques known in the arts.

In one embodiment of an *in vivo* assay, a cell expressing *Wnt5a* is contacted with an agent to be tested. The level of *Wnt5a* expression or activity is then determined using an assay known in the art. These assays may include but are not limited to Northern blot analysis, enzyme activity, quantitative PCR, Western blot analysis, *etc.* As would be appreciated by one of skill in this art, experiments designed to screen for agents directed against *Wnt5a* may include proper positive and/or negative controls. The experiment may also include testing a particular agent a several difference concentrations in the range of about 1 nM to about 100 mM, preferably about 1 nM to about 1mM, more preferably about 1 nM to about 100 μM.

In one preferred embodiment, the cells used in the screening method are skin cells, more preferably malignant melanoma cells. In certain embodiments, the cells or cell line are genetically engineered to express *Wnt5a*. In certain embodiments, the cells are malignant melanoma cells that did not express *Wnt5a* naturally but have been genetically engineered to express *Wnt5a*. Preferred embodiments of such cells and cell lines are described below in the Examples.

Inventive methods of detecting whether a compound inhibits *Wnt5a* may include an assay which assesses the ability of the cells to "chew through", digest, or migrate through extracellular matrix as described below in the Examples. Assays of this type may include, but are not limited to, the scratch assay, and the Boyden chamber assay. A cell that overexpresses *Wnt5a* may be able to digest or migrate through extracellular matrix in its search for media or nutrients. Agents that inhibit such a cell's ability to digest extracellular matrix and/or may be inhibiting the activity of *Wnt5a* may be useful in the treatment malignant melanoma expressing *Wnt5a*. In a preferred embodiment, the agent reduces the ability of the cell to digest or migrate through extracellular by at least about 50% when

compared to cell that were not contacted with the agent, more preferably by at least about 75%, and most preferably by at least about 90%.

In certain other embodiments, cell morphology or cytoskeletal organization may be used to assess the effect of an agent on cells expressing *Wnt5a*. The cells may be contacted with various concentrations of the agent with a control plate of cells contacted with no agent. The shape of the cells, number of attachments of each cell to the plate, and/or the organization of actin filaments may be assessed to determine the effect of the agent on the cells. In other embodiments, downstream signaling molecules in the *Wnt5a* pathway are analyzed to determine the effect of the added agent. In one embodiment, the phosphorylation of protein kinase C is used to determine the effect of the agent.

In other embodiments, agents may be screened for their ability to inhibit or knock out the *Wnt5a* pathway as shown in Figure 6. In one embodiment, agents may be screened for their ability to block the binding of WNT5A to its receptor, frizzled 5. An agent able to block this binding interaction could possibly attenuate or reverse the phenotypes that increased WNT5A would normally produce, such as increased cell movement an invasiveness.

These and other aspects of the present invention will be further appreciated upon consideration of the following Examples, which are intended to illustrate certain particular embodiments of the invention but are not intended to limit its scope, as defined by the claims.

Examples

Example 1-Molecular Classification of Cutaneous Malignant Melanoma by Gene Expression Profiling

We have proposed that a discrete and previously unrecognizable cancer taxonomy can be identified by viewing the systematized data from gene expression experiments (Bittner *et al. Nature* 406:536-540, 3 August 2000; incorporated herein by reference). However, for melanoma, inherent or technically induced variation could obscure such a classification as its appearance is very similar between patient samples and, in contrast to haematologic cancers (Golub *et al.* "Molecular classification of cancer, class discovery and class prediction by gene expression monitoring" *Science* 286:531-537, 1999; Alizadeh *et al.* "Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling" *Nature* 403:503-511, 2000; each of which is incorporated herein by reference), it has few known recurring genetic changes. To explore this question, we gathered expression profiles for 38 samples, including 31 melanomas and 7 controls (Table 1). Total messenger RNA was isolated directly from melanoma biopsies or tumor cell cultures, prepared fluorescent complementary DNA from the message and hybridized them to a microarray containing probes for 8,150 cDNAs (representing 6,971 unique genes), obtaining quantitative and comparative measurements for each gene.

The tumor cell mRNA was compared with a single reference probe, providing normalized measures of the expression of each gene in each sample relative to the standard. Analysis of the normalized expression across all genes between samples provided a measure of the overall difference in expression pattern between samples. Similarly, the orthogonal analysis of linear covariance between pairs of genes across all samples provided a measure of the similarity of behavior of the genes studied.

Figure 1 shows the integration of several analytical methods to visualize the overall expression pattern relationships between cutaneous melanoma tumor samples. Using a matrix of Pearson correlation coefficients from the complete pair-wise comparison of all experiments (Bittner *et al.* "Data analysis and integration of steps and arrows" *Nature Genet.* 22:213-215, 1999; incorporated herein by reference), the 31 melanoma experiments are displayed as a hierarchical clustering dendrogram (Khan *et al.* "Gene expression profiling of alveolar rhabdomyosarcoma with cDNA microarrays" *Cancer Res.* 58:5009-5013, 1998; Eisen *et al.* "Cluster analysis and display of genome-wide expression patterns" *Proc. Natl. Acad. Sci. USA* 95:14863-14868, 1998; each of which is incorporated herein by reference) and as a three-dimensional multidimensional scaling (MDS) plot (Khan *et al.* "Gene expression profiling of alveolar rhabdomyosarcoma with cDNA microarrays" *Cancer Res.* 58:5009-5013, 1998; Everitt, B. *Applied Multivariant Data Analysis.* (Oxford Univ. Press, New York, 1992); incorporated herein by reference). The MDS plot displays the position of each tumor sample in three-dimensional Euclidean space, with the distance between experimental samples reflecting their approximate degree of correlation (Khan *et al.* "Gene expression profiling of alveolar rhabdomyosarcoma with cDNA microarrays" *Cancer Res.* 58:5009-5013, 1998; Everitt, B. *Applied Multivariant Data Analysis.* (Oxford Univ. Press, New York, 1992); incorporated herein by reference). The analysis included all genes meeting a minimum level of expression in each hybridization. We also employed a non-hierarchical clustering algorithm (termed cluster affinity search technique; CAST) (Ben-Dor *et al.* "Clustering gene expression patterns" *J. Comput. Biol.* 6:281-297, 1999;incorporated herein by reference) to define experimental clusters. The resulting hierarchical dendrogram of the 31 melanoma samples (Fig. la) demonstrates that 19 samples are tightly clustered at the bottom of the dendrogram in the area of highest similarity. Likewise, the non-hierarchical

CAST algorithm identified the identical major cluster 19 melanomas. This cluster is also a compact, readily separable grouping based on its overall similarity of expression pattern viewed by MDS (Fig. 1b).

There is no single established method to estimate the significance of an observed degree of relationship obtained by cluster prediction techniques (Golub *et al.* "Molecular classification of cancer, class discovery and class prediction by gene expression monitoring" *Science* 286:531-537, 1999; Bittner *et al.* "Data analysis and integration of steps and arrows" *Nature Genet.* 22:213-215, 1999; each of which is incorporated herein by reference). Accordingly, we used two independent approaches to test the validity of our cluster prediction of the 19-element cluster. The first approach (Fig. 1c) examines the power of individual genes to discriminate the major cluster of 19 from the remaining samples by examining the frequency of strong classifier genes compared to the expected frequency of such genes if expression is randomly variable, and to the frequency of strong classifiers in random partitions of the same samples into new groupings of 19 and 12 (Ben-Dor *et al.* "Class Discovery in Gene Expression Data" *Proceeding RECOMB 2001*, pp. 31-38, 2001; incorporated herein by reference). The non-randomness of the cluster results is evident. Specifically, many genes have expression patterns that differ strongly between the initial sample clusters and thus serve as good classifiers (Fig. 1c, red triangles). However, expression patterns are not readily found which classify the samples when they are grouped into random partitions of the same size (Fig. 1c, blue lines). Accordingly, in randomly formed clusters, expression behavior is essentially indistinguishable from truly random behavior of genes relative to these clusters (Fig. 1c, compare blue lines with open circles).

The second approach we used to test the validity of the cluster predictions is based on evaluating cluster membership after introducing random perturbations to the data set. For

each sample, the log-ratio of each gene was perturbed by the introduction of random gaussian noise with the mean equal to 0 and the standard deviation equal to 0.15 (an estimate of variation derived by computing the median standard deviation of the log-ratios for single genes across all 31 samples). Hierarchical clustering was then performed on the perturbed data set and a comparison made between the original tree (Fig. 1a) and the perturbed tree. Comparisons involved cutting the original and perturbed trees into k clusters followed by computing the proportion of paired samples clustering together in the original tree that did not cluster together in the perturbed tree (we refer to this measure as a weighted proportion of discrepant pairs because it gives more weight to larger clusters). The comparison was repeated over multiple perturbed data sets for each possible cut in the original tree ($k = 2, 3, \ldots, 30$). For a given k, the weighted proportion of discrepant pairs was then averaged over the perturbed data sets resulting in the identification of weighted average discrepant pairs ($WADP_k$; see Supplementary Information).

Clusters that result from cutting the original tree into 9 or fewer groups are very reproducible (Fig. 1d). It is noteworthy that the rise in $WADP_k$ almost exactly coincides with the division of the major 19-element cluster into smaller sub-clusters. These results strongly support the view that the major cluster of melanoma samples identified in this study represents a bona fide and highly reproducible grouping.

We then performed statistical tests to determine whether any clinical or tumour cell characteristics were specifically associated with the clustered group. Tests for associations between the major cluster of 19 samples and the remaining 12 melanoma samples were performed for several *in vivo* variables, including sex, age, biopsy site, Breslow thickness, Clark's level and survival. There was no statistically significant association between the cluster group and any clinical variable. There were also no significant associations with the

in vitro variables, including p16 or β-catenin mutation status, *in vitro* pigmentation and cell passage number (see Supplementary Information).

We included two pairs of specimens derived from the same patient in this sample set. These are M92-001 and M93-007 (two different samples from the same individual, surgically removed one year apart), and TD-1376-3 and TC-1376-3 (the biopsy sample and a cell culture of the same tumour carried three passages *in vitro*). Although there was no significant association between cell passage number and cluster group ($P = 0.857$, see Supplementary Information), the TD-1376-3/TC-1376-3 pair were included to serve as another control for the effects of cell culture. Remarkably, of the 465 pairwise comparisons among the melanoma samples, the pairs TD-1376-3/TC-1376-3 and M92-001/M93-007 are the second and third most highly correlated pairs of samples, with nearly identical correlation coefficients (Fig. 1b).

On the basis of the linear correlation of global gene expression in Fig. 1, Figs 2 and 3 illustrate the approach we have used to guide 'gene cluster' interpretation empirically. Fig. 2a depicts our statistical method for extracting a 'weighted list' of individual genes whose variance of change across all experiments correctly defines the boundary of a given sample cluster (for details see Supplementary Information). Fig. 2b displays the list of genes with the most power to define the major melanoma cluster of 19 samples (Fig. 1a and b) in rank order along the vertical axis. The samples are ordered along the horizontal axis by cluster inclusion, and data are presented graphically as coloured images with the colour saturation directly proportional to the magnitude of the measured gene expression ratio (brightest red, highest *R/G* ratio; black squares, *R/G* ratio = 1; brightest greens, lowest *R/G* ratio). The complete list of genes discriminating the major cluster is in the Supplementary Information.

The weighted gene list can also be used to guide analysis of the larger gene expression data set. Figure 3a displays all data from the cutaneous melanoma samples in this study as a coloured image with genes ordered along the vertical axis by similarity of expression pattern (after Eisen *et al.* "Cluster analysis and display of genome-wide expression patterns" *Proc. Natl. Acad. Sci. USA* 95:14863-14868, 1998; incorporated herein by reference). However, rather than basing analysis of this large (>300,000 elements) data set entirely on visual selection, we used genes from the weighted list to index gene cluster selection. Figure 3b-e illustrates this approach using four genes from the 'weighted list' in Fig. 2b (MART-1, CD63, tropomyosin and WNT5A), to interrogate the entire gene expression data set represented in Fig. 3a.

Table 1 Summary of melanoma cases by cluster designation

Case no.	Sex/Age	Biopsy site	Passage no. (Biopsy)	p16 mutation status*	Invasive ability†	Vasulogenic mimicry‡	Gel contraction§	Cell motilit!!	Scratch wound (%)¶
Melanoma primary cluster									
UACC-502	M/69	Cervical node	3	Deleted	2.8 ±. 01%	-	ND	ND	37
M92-001	F/43	Ankle	2	Deleted	3.0 ± 0.5%	-	ND	76.8 ±2. 96	22
A-375	F/54	Skin	ND	Mutation	2.8 ± 0.2%	-	ND	67.80 ± 4.40	26
M91-054#	M/45	Axill. lymph node	3	WT	#	#	#	ND	30
UACC-1256	F/67	Thigh femoral node	9	Deleted	ND	ND	ND	ND	ND
M93-007	F/43	Ankle	3	Deleted	2.6 ± 0.1%	-	-	ND	12
UACC-091	M/52	Unk	7	Deleted	2.1 ± 0.2%	-	-	ND	11
UACC-1273	M/50	Axill. lymph node	16	Mutation	2.5 ± 0.3%	-	-	ND	13
TD-1730	M/55	Thyroid lobe	Biopsy	ND	ND	ND	ND	ND	ND
TD-1638	M/49	Paraspinous	Biopsy	ND	ND	ND	ND	ND	ND
TD-1720	M/29	Shoulder	Biopsy	ND	ND	ND	ND	ND	ND
TD-1348	M/44	Axill. lymph node	Biopsy	ND	ND	ND	ND	ND	ND
UACC-1022	F/53	Chest wall	13	WT	2.9 ± 0.1%	-	-	ND	63
TC-1376☼	M/30	Distal ileum	3	ND	ND	ND	ND	ND	21
TD-1376☼	M/30	Distal ileum	Biopsy	ND	ND	ND	ND	ND	ND
UACC-2534	M/68	Abdomen	7	Deleted	3.2 ± 0.02%	-	ND	ND	7
UACC-383	M/69	Thigh femoral node	29	Deleted	2.3 ± 0.2%	-	ND	70.40 ± 5.27	35
UACC-457	FUkn	Unk	19	WT	3.1 ± 0.2%	-	ND	12.80 ± 0.05	ND
UACC-3093	M/75	Axill. lymph node	4	WT	ND	ND	ND	40.30 ± 2.00	24
Melanoma non-clustered									
UACC-930	F/35	Sm. bowel	4	WT	4.8 ± 0.3%	±	-	ND	50
M93-047	F/75	Axill. lymph node	3	Mutation	10.7 ± 0.03%	+	+	ND	75

Table 1 Summary of melanoma cases by cluster designation

Case no.	Sex/Age	Biopsy site	Passage no. (Biopsy)	p16 mutation status*	Invasive ability†	Vasulogenic mimicry‡	Gel contraction§	Cell motilit!!	Scratch wound (%)¶
UACC-2973	M/37	Axill. lymph node	5	ND	ND	ND	ND	ND	48
UACC-903	M/25	Back	14	Deleted	3.8 ± 0.3%	+	-	ND	91
TC-F027	M/30	Rt. chest wall	6	ND	ND	ND	ND	ND	91
UACC-1097	M/56	Rectus muscle	6	Mutation	ND	ND	ND	ND	34
UACC-647**	M/32	Axill. node	14	WT	3.8 ± 0.1%	+	±	ND	55
UACC-1012	M/54	Neck	3	ND	4.9 ± 0.1%	ND	ND	122.00 ± 11.30	54
UACC-827	F/32	Rt. breast	16	Mutation	ND	ND	ND	ND	32
WM1791C	Unk	Ukn	52	ND	4.6 ± 0.3%	+	ND	141.00 ± 11.40	71
HA-A	F/Ukn	Ukn	19	ND	3.9 ± 0.5%	±	ND	211.00 ± 12.40	62
UACC-1529	M/48	Axill. lymph node	13	Mutation	4.2 ± 0.5%	+	-	ND	ND
Uveal melanoma samples									
CCM-1A	Unk	Primary	25	ND	2.2 ± .01%	-	-	ND	ND
C918	F/60	Primary	15	ND	12.9 ± 03%	+	+	ND	ND
MUM-2C	M	Liver metastases	8	ND	2.0 ± 0.1%	-	-	ND	ND
MUM-2B	M	Liver metastases	8	ND	13.3 ± 0.6%	+	+	ND	ND
Control samples									
Nil. C (fibroblast); UACC-3149 (ovarian adenocarcinoma); MCF-10A (breast epithelium); CRL-1634 (fibroblast); SRS-3 (cell culture variant); SRS-5 (cell culture variant);RMS-13 (rhabdomyosarcoma)									

* Mutation status of indicated samples for p16 obtained by sequencing. Deleted, homozygous. Supplementary Information includes the specific mutations in p16 for each sample tested. Samples were also sequenced for β-catenin. No example of β-catenin mutation was observed.
†Ability to invade a defined basement matrix. P = 0.0055; t-test for two populations.
‡Tube forming ability at 5 days in a three-dimensional matrigel matrix.
§ Ability to contract floating collagen 1 gels at 5 days as compared to HT-1080 fibrosarcoma cells (Maniotis *et al.* "Vascular channel formation by human melanoma cells *in vivo* and *in vitro*: vasculogenic mimicry" *Am. J. Pathol.* 155:739-752, 1999; incorporated herein by reference)
!!Migration rates expressed in μm per day. Mean from eight experiments ± s.d. (P = 0.0063; t-test for two populations). Rates below 100 μm per day completely segregates in the melanoma primary cluster.
¶ Ability to close in vitro scratch wound at 24 h. Photographs of the wound were measured and percentage wound closure determined (Silletti *et al.* "Autocrine motility factor and the extracellular matrix I. Coordinate regulation of melanome cell adhesion, spreading and migration involves focal contact reorganization" *Int. J. Cancer* 76:120-128, 1998; incorporated herein by reference) (P < 0.00002, t-test for two populations).
# M91 -054 was the only sample that demonstrated a mixed phenotype in culture with both an epitheloid population and a more fibroblastic population. Vasculogenic mimicry and gel contraction were only observed in the epitheloid population. Scratch assay resulted in 30 % closure after 24 h for both populations.
☼ TC-1376 mRNA was isolated after short term (3 passage) culture of the biopsy sample from the patient TD-1376 allowing the effects of short term culture on the expression profile to be observed.
** UACC-647 cells form extensive cord-like networks by 5 days.

Finally, in parallel to our microarray analysis of cutaneous melanoma, we studied a series of uveal melanoma specimens characterized for properties related to metastasis, including invasive ability and vasculogenic mimicry *in vitro* (Maniotis *et al.* "Vascular channel formation by human melanoma cells *in vivo* and *in vitro*: vasculogenic mimicry" *Am. J. Pathol.* 155:739-752, 1999; incorporated herein by reference). These samples were hybridized pairwise, directly comparing highly invasive cells to their less invasive counterparts. We examined the pattern of gene expression in these phenotypically

characterized cells with respect to the weighted discriminator list (Fig. 2b) that defines the major cluster of 19 cutaneous melanomas. Strikingly, genes expressed in common in the highly invasive uveal melanoma cells (Fig. 2b, inset) were strongly anti-correlated with the same gene from the major cluster of cutaneous melanoma samples (Fig. 2b). This observation, coupled with the known biological function of genes within the weighted list, indicated that specimens assigned within the major cutaneous melanoma cluster (Fig. 1a, b) would have reduced motility and reduced invasive ability as they have down-regulation of genes related to cell spreading or migration, including formation of focal adhesions (Adams "Characterization of cell-matrix adhesion requirements for the formation of fascin microspikes" *Mol. Biol. Cell* 8:2345-2363, 1997; Scott *et al.* "pp125FAK in human melanocytes and melanoma: expression and phosphorylation" *Exp. Cell Res.* 219:197-203, 1995; each of which is incorporated herein by reference). Specific genes with reduced expression in the major cluster included integrin ß1 (Jannji *et al.* "Autocrine TGF-beta-regulated expression of adhesion receptors and integrin-linked kinase in HT-144 melanoma cells correlates with their metastic phenotype" *Int. J. Cancer* 83:255-262, 1999; Hieken *et al.* "Betal integrin expression in malignant melanoma predicts occult lymph node metastases" *Surgery* 118:669-673, 1995; each of which is incorporated herein by reference), integrin ß3 (Van Belle *et al.* "Progression-related expression of beta3 integrin in melanomas and nevi" *Hum. Pathol.* 30:562-567, 1999; incorporated herein by reference), integrin α1 (Hieken *et al.* "Betal integrin expression in malignant melanoma predicts occult lymph node metastases" *Surgery* 118:669-673, 1995; incorporated herein by reference), syndecan 4 (Woods *et al.* "Syndecan-4 binding to the high affinity heparin-binding domain of fibronectin drives focal adhesion formation in fibroblasts" *Arch. Biochem. Biophys.* 374:66-72, 2000; incorporated herein by reference) and vinculin (Helige *et al.* "Interrelation of motility, cytoskeltal

organization and gap junctional communication with invasiveness of melanocytic cells *in vitro*" *Invasion Metastasis* 17:26-41, 1997; incorporated herein by reference) (Figs 2 and 3; see Supplementary Information). In samples outside the major cluster increased expression of fibronectin is particularly interesting. With other reports (Maung *et al.* "Requirement for focal adhesion kinase in tumor cell adhesion" *Oncogene* 18:6824-6828, 1999; Silletti *et al.* "Autocrine motility factor and the extracellular matrix I. Coordinate regulation of melanome cell adhesion, spreading and migration involves focal contact reorganization" *Int. J. Cancer* 76:120-128, 1998; each of which is incorporated herein by reference), this observation indicates that these cells are induced to secrete this pro-migratory molecule, consistent with an important role for focal contacts in modulating melanoma cell motility.

We then directly tested the prediction from the array results that cell spreading and migration could be discordant between melanoma cluster groups. Cutaneous melanomas (assigned either in or out of the major cluster) were characterized using a series of cellular assays applied to test cell motility and invasiveness (Table 1, Fig. 4). Figure 4 illustrates the discordance of cutaneous melanoma samples within the major cluster and those outside this group. As predicted from the analysis of their gene expression patterns, melanomas within the major cluster had reduced motility ($P = 0.0063$), invasive ability ($P = 0.0055$) and vasculogenic mimicry in comparison with melanomas outside the major cluster (Table 1).

The patient population in this study had a uniformly poor prognosis, and neither typical clinical factors (for example, age, sex, biopsy site) nor *in vitro* characteristics (for example, passage number) provide strong correlation with clinical outcome, or expression information (see Supplementary Information). In contrast, molecular classification of these tumors on the basis of gene expression (Fig. 1, Table 1) could identify a previously undetected subtype of this cancer. The analyses described here were not designed to address

the relationship of gene expression profile and clinical outcome in melanoma patients, and thus the clinical relevance of our observed subgrouping awaits further analysis. However, survival information was available on 15 patients, and the results, though not statistically significant, are of interest. Three deaths occurred out of 10 patients in the tight cluster of 19 while 4 deaths occurred out of 5 patients in the remaining group (log-rank *P*-value = 0.135). Our results indicate melanoma will provide a unique opportunity to study a homogeneous group of patients to determine if gene expression patterns predict prognosis or therapeutic response in settings where we cannot currently determine who is most at risk for rapid disease progression and death.

Finally, classification of melanoma on the basis of gene expression patterns is possible, despite the prevailing view that the 'taxonomy' of this disease falls in a continuous spectrum lacking discernible entities. Our data show that melanoma is a useful model to identify genes critical for aspects of the metastatic process, including tumour cell motility and the ability to form primitive tubular networks that may contribute to tumour perfusion. The extent to which melanoma samples can be clinically subdivided by expression patterns remains to be elucidated. However, our identification of genes 'weighted' for their ability to discriminate a subset of melanomas should provide a sound molecular basis for the dissection of other clinically relevant subsets of this tumur.

Methods

Samples

Cultured cells were collected and mRNA isolated as described (Khan *et al.* "DNA Microarray technology: the anticipated impact on the study of human disease" *Biochim. Biophys. Acta*

1423:17-28, 1999; www.nhgri.nih.gov//DIR/microarray; each of which is incorporated herein by reference). Samples underwent a series of controls for quality of mRNA, labeling and hybridization, as well as sample integrity (including genotyping DNA from all samples with five dinucleotide markers from four different chromosomes to insure individuality). The entire coding sequence of the p16 gene and exon 3 of the ß-catenin genes was sequenced to assess the mutation status of all available samples (see Supplementary Information). The biopsy tumour specimens used in this study were obtained with Institutional Review Board approval and clinical information is provided in the Supplementary Information. Biopsies were debrided, dissected into small pieces and frozen in liquid nitrogen. Frozen specimens were immediately placed into TRIzol Reagent (Gibco BRL), homogenized and mRNA isolated as described (Khan *et al.* "DNA Microarray Technology: The Anticipated Impact on the Study of Human Disease" *Biochim. Biophys. Acta* 1423:17-28, 1999; www.nhgri.nih.gov/DIR/microarray; each of which is incorporated herein by reference).

Microarrays

The 8,150 human cDNAs used in this study were obtained under a Cooperative Research and Development Agreement with Research Genetics and 6,912 were verified by sequence. This set of cDNAs is part of a larger collection (Khan *et al.* "Gene expression profiling of alveolar rhabdomyosarcoma with cDNA microarrays" *Cancer Res.* 58:5009-5013, 1998; Duggan *et al.* "Expression profiling using cDNA microarrays" *Nature Genet.* 21:10-14, 1999; www.nhgri.nih.gov/DIR/microarray; each of which is incorporated herein by reference). On the basis of the Unigene build of 9 March 2000 (http://www.ncbi.nlm.nih.gov/UniGene/build.html), the 8,150 cDNAs represent 6,971 unique genes in this melanoma array. All clones were confirmed by resequencing if necessary.

Microarrays were hybridized, scanned and image analysis performed as described (Khan *et al.* "Gene expression profiling of alveolar rhabdomyosarcoma with cDNA microarrays" *Cancer Res.* 58:5009-5013, 1998; Khan *et al.* "DNA Microarray technology: the anticipated impact on the study of human disease" *Biochim. Biophys. Acta* 1423:17-28, 1999; www.nhgri.nih.gov/DIR/microarray; each of which is incorporated herein by reference). The raw data from the microarray is shown in Appendix A, a Microsoft Excel Worksheet, which has been included on a CD-ROM submitted with this application and is incorporated herein by reference.

Statistical methods

Detailed information on all statistical methods is in the Supplementary Information. Agglomerative hierarchical clustering of the 31 melanomas on the basis of their gene expression profiles was performed as described (Khan *et al.* "Gene expression profiling of alveolar rhabdomyosarcoma with cDNA microarrays" *Cancer Res.* 58:5009-5013, 1998; Bittner *et al.* "Data analysis and integration of steps and arrows" *Nature Genet.* 22:213-215, 1999; each of which is incorporated herein by reference), to investigate relationships between tumour samples. Average linkage was used, as well as a dissimilarity measure of one minus the Pearson correlation coefficient of log ratios. The cutoff employed to obtain the observed partitioning was 0.54. The MDS was performed using an implementation of MDS in the MATLAB package. A non-hierarchical clustering algorithm (Ben-Dor *et al.* "Clustering gene expression patterns" *J. Comput. Biol.* 6:281-297, 1999; incorporated herein by reference) was used to define experimental clusters. This approach takes a graph theoretic approach, and makes no assumptions on the similarity function or the number of clusters sought.

To generate the weighted gene list, cluster compaction and separation were evaluated. For a given clustering result, $n_1 = 19$ and $n_2 = 12$, the discriminative weight of each gene $w = d_B/(k_1 d_{w1} + k_2 d_{w2} + \alpha)$; where d_B is the centre-to-centre distance (between cluster Euclidean distance), d_{w1} is the average Euclidean distance among all sample pairs within cluster i, k, = $t_1/(t_1 + t_2)$ for a total of t; sample pairs in cluster i, and α is a small constant (0.1 in our study) to prevent the zero denominator case (Fig. 2a). Genes may then be ranked on the basis of w.

***In vitro* biological assays**

Floating collagen lattices were prepared and used to test selected cell lines for their ability to deform the gels as described (Maniotis *et al.* "Vascular channel formation by human melanoma cells *in vivo* and *in vitro*: vasculogenic mimicry" *Am. J. Pathol.* 155:739-752, 1999; Table 1 legend). Samples were also tested for their ability to migrate into an *in vitro* scratch wound as described (Tamura *et al.* "Inhibition of cell migration, spreading and focal adhesions by tumor suppressor PTEN" *Science* 280:1614-1617, 1998; incorporated herein by reference). Cells were stained with Giemsa, a digital micrograph of the region was prepared and the stained area as a percent of total area in the scraped and open sub-regions was estimated by a thresholding procedure using IPLabs Spectrum (Scanalytics, Vienna, Virginia) software. Results in Table 1 represent data from 24 h after plating on coverslips treated with fibronectin (FN; 10 $\mu g\ ml^{-1}$; Tamura *et al.* "Inhibition of cell migration, spreading and focal adhesions by tumor suppressor PTEN" *Science* 280:1614-1617, 1998; incorporated herein by reference).

Examples of tubular network formation (associated with vasculogenic mimicry) could be observed following seeding of cell lines onto three-dimensional gels of polymerized Matrigel or Type 1 collagen (Collaborative Biochemical) as described (Maniotis *et al.*

"Vascular channel formation by human melanoma cells *in vivo* and *in vitro*: vasculogenic mimicry" *Am. J. Pathol.* 155:739-752, 1999; Table 1).

Table 1 lists results from high throughput screening for cell migration as the radial dispersion of cells from an initial confluent monolayer of 2,000 melanoma cells deposited within a 1.0 mm circular area on glass surfaces precoated with FN (100 μg ml^{-1}; Berens *et al.* "The role of extracellular matrix in human astrocytoma migration and proliferation studied in a microliter scale assay" *Clin. Exp. Metastasis* 12:405-415, 1994; Giese *et al.* "Contrasting migratory response of astrocytoma cells to tenascin mediated by different integrins" *J. Cell Sci.* 109:2161-2168, 1996; each of which is incorporated herein by reference).

Selected cell lines were tested for their ability to invade a defined basement membrane matrix. Tumor cells (1 x 10^5) were seeded into the upper wells of the membrane invasion culture system (MICS) chamber (Hendrix *et al.* "A simple quantiative assay for studing the invasive potential of high and low human metastatic variants" *Cancer Lett.* 38:137-147, 1987; incorporated herein by reference) onto collagen/laminin/gelatin-coated (Sigma) polycarbonate membranes containing 10-μm pores (Osmonics, Livermore, California) containing lx Mito+ Serum Extender (Becton Dickinson). After 24 h of incubation at 37°C, the cells that invaded each membrane were collected, stained and counted as described (Hendrix *et al.* "Role of intermediate filaments in migration, invasion and metastasis" *Cancer Metastasis Rev.* 15:507-525, 1996; incorporated herein by reference). Percent invasion was corrected for proliferation and calculated as (total number of invading cells/ total number of cells seeded) x 100.

Supplement I - Statistical Methods for Clustering of Gene Expression Data and

Validation of Cluster Predictions

OVERVIEW:

To fully appreciate the expression patterns derived from large number of cDNA microarrays and their relationship between melanoma tumor samples, several statistical methods were integrated as follows,

a. Multidimensional scaling (MDS) method was employed in order to visualize the similarity between samples, and a hierarchical clustering dendrogram was produced by an implementation of the average-linkage clustering algorithm,

b. The clustering results were further verified by a non-hierarchical algorithm, CAST (Ben-Dor *et al. J. Comput. Biol.* 6:281-297, 1999; incorporated herein by reference),

c. In order to determine the tightness and the statistical significance of the clusters derived from various methods, two independent approaches were assembled to validate the prediction. One, $WADP_k$ method, is sensitivity analysis of the noise perturbation to the data set. The other one is based on comparing the discrimination power observed for genes in the data to that expected in random data. This is accomplished using TNoM scoring.

d. After confirming the clustering result, each gene was weighted based on their discriminative ability for the clusters derived from previous method.

In the following section, detailed descriptions of the methods listed in Steps 3 to 4 will be presented. For some of the more standard methods, such as MDS, average-linkage methods, and CAST, we refer readers to the literature (Ben-Dor *et al. J. Comput. Biol.* 6:281-297, 1999; Eisen *et al. Proc. Natl. Acad. Sci. USA* 95:14863-14868, 1998; Everitt *Cluster Analysis*

(London: Edward Arnold), 1993; each of which is incorporated herein by reference). Since not all genes were readily detectable by the array method, a subset of the total number of surveyed genes was analyzed in all cases. A set of 3613 genes was chosen for analysis. The genes were chosen by an empirically derived set of criteria requiring an average mean intensity above background of the least intense signal (Cy3 or Cy5) across all experiments >2000 arbitrary units, and an average spot size across all experiments of >30 pixels. To avoid distortions of the data resulting from ratios where the signal in one channel is large, and the signal in the other channel is undetectable, ratios higher than 50 or lower than 0.02 were truncated to 50 or 0.02 for these analyses.

Description of the WADP$_k$ method for testing the validity of cluster predictions

Hierarchical clustering of the 31 melanoma samples was performed, resulting in a dendrogram (Fig. 1b). Although the dendrogram gives insights about the similarity and relatedness among samples, it does not indicate robustness to variability associated with the assay sampling, etc. In order to draw valid conclusions about the clustering structure present in the data, it is necessary to investigate how variability affects the results of the cluster analysis. To this end, we developed and implemented a method that determines the reproducibility of given levels of cluster structure within the dendrogram under the condition of added noise. The method is described below.

First, cut the original dendrogram at a height that results in k clusters and let N_k denote the number of clusters containing 2 or more elements. Let M_i represent the number of pairs of elements in the i^{th} of the N_k clusters. Next, perturb the data by adding to every log-ratio of each sample an independent random deviate generated from the $N(0,\square)$ distribution. Cluster the perturbed data and cut the resulting dendrogram at a height that again results in k clusters.

For the M_i pairs of elements in the i^{th} original cluster, record the number of those pairs, D_i that do not remain together in the clustering of the perturbed data. Next, calculate the overall discrepancy rate for the clustering: $(D_1 + D_2 + \ldots + D_{N_k})/(M_1 + M_2 + \ldots + M_{N_k})$. This overall discrepancy rate is a weighted average of the N_k cluster-specific discrepancy rates (*i.e.*, D_i/M_i, for i = 1, 2, ..., N_k), with weights proportional to the number of pairs in individual clusters. Finally, repeat the calculations over many perturbations of the original data set and report the average overall discrepancy rate (termed the Weighted Average Discrepant Pairs for k clusters, or $WADP_k$). The above procedure is repeated for all possible cuts of the original dendrogram and $WADP_k$ is plotted versus k. Minima of the WADP curve are interpreted as indicating reproducible levels of structure.

The parameter σ represents the noise standard deviation inherent to the system. As mentioned above, the noise is composed of—at the least—assay variability and sampling variability. σ is unknown and must be estimated. The method we use for estimating σ is to compute the variance of the log-ratio of each gene across all samples. We then use the median of the empirical distribution of these variances as an estimate of σ^2 It may be more appropriate to use a smaller value (say the tenth percentile of the empirical distribution), if it were believed that a large percentage of genes present on the array were truly differentially expressed within the population of samples hybridized.

Description of the TNoM method for the cluster significance based on random partition.

Threshold number of misclassification, or *TNoM score*, is a simple threshold-based method that uses a given expression level, for a given gene, to predict the cluster label of a given test sample. In the present study, we have 31 samples form 2 groups. Therefore, we can label the samples by l_i , $i = 1, \ldots, m$, where $l_i \in \{0,1\}$ and $m = 31$. For the kth gene, let $\langle x_i, l_i \rangle_k$

be its expression pattern (or ratios in this study) and corresponding cluster labels. A threshold function is defined as,

$$f_{h,a}(x) = \begin{cases} a, & \text{if } x < h \\ 1-a & \text{otherwise} \end{cases}$$

where h is a threshold value, and $a \in \{0,1\}$. For a given h and a we can assign the label $f_{h,a}(x_i)$ to the ith sample. The number of misclassifications entailed by this scheme is,

$$e = \sum_{i=1}^{m} \left| l_i - f_{h,a}(x_i) \right|$$

The TNoM score for the kth gene, s_k, is defined as the minimum error achieved over all possible choices of h and a,

$$s_k = \min_{h,a} \left(\sum_{i=1}^{m} \left| l_i - f_{h,a}(x_i) \right| \right)$$

The minimization step is accomplished by exhaustively searching all $2(m+1)$ possibilities.

To examine the significance of groups derived by clustering algorithm, we used three steps. First, we evaluated TNoM scores for all genes found in the data set. Then, the number of genes that have TNoM score less than or equal to s, for $s = 0, \ldots, 12$ (where 12 is the maximum misclassifications any classification rule may commit) was listed. Next, we randomly assigned cluster labels to all samples to form two arbitrary groups of 19 and 12 samples. The TNoM score was again evaluated for each gene. A list of the number of genes that have TNoM score less than or equal s was similarly obtained. We repeated this process 50 times to observe random fluctuations and their range of scores. Finally, the expected number of genes resulting in s or fewer misclassifications under the assumption of perfect random gene expression patterns can be calculated (Ben-Dor *et al.*, submitted for publication). As expected, the value produced by the 50 random sampling is close to those

produced by the theoretical rigorous calculation. The significance of the suggested clusters is reflected in the overabundance of genes with low TNoM scores. More precisely, a meaningful partition will produce far more genes with low TNoM scores than a random one.

Description of the weighting method based on gene's discriminative ability.

The clustering algorithms described in the text produced one tightly bonded cluster of $n_1 = 19$ samples, and we assume the rest of $n_2 = 12$ samples form another cluster. For a given two-cluster setting, a discriminative weight for each gene can be evaluated by,

$$w = d_B / (k_1 d_{w_1} + k_2 d_{w_2} + \alpha)$$

where d_B is the center-to-center distance (between cluster Euclidean distance), d_{w_i} is the average Euclidean distance among all sample pairs, total of t_1 and t_2 sample pairs for cluster 1 and 2, respectively, and $k_1 = t_1/(t_1+t_2)$, and $k_2 = t_2 / (t_1+t_2)$. α is a small constant (0.1 in our study) to prevent zero denominator case. Genes may then be ranked on the basis of w. The equation for weight w is not only designed to evaluate discriminative ability for single gene, but also capable of evaluate discriminative ability for 2 or more genes together. If you do not assume the second group of samples to be a tight cluster you can drop the d_{w_2} term.

Supplement II - Statistical Analysis of Clinical and Culture Characteristics of Melanoma Clusters

SUMMARY REPORT:

Thirty-one tissue specimens were clustered using the Bioclust clustering algorithm (see text), resulting in one tight cluster of 19 specimens (Group A) and 12 specimens that showed no specific clustering pattern (Group B). Statistical tests were performed to

determine whether any clinical or tumor cell characteristics were specifically associated with cluster group. For categorical variables we created a contingency table and used Fisher's exact test to compute a p-value (the Chi-square test was not used because each table had at least one expected cell frequency less than 5). For continuous and ordered variables, we used the Wilcoxon two-sample (rank-sum) test, a non-parametric alternative to the two-sample *t* test. Tests were performed in S-plus 4.5 and StatXact 3.1.

The two groups consisted of the following patient IDs:

Group A				Group B		
M93-007	M91-054	UACC091	UACC502	HA-A	UACC827	UACC1529
UACC1256	UACC127	UACC253	M92-001	UACC647	UACC930	M93-047
UACC457	UACC383	UACC309	A-375	UACC2837	TC-F027	WM1791C
UACC1022	TD1376-3	TD1683	TD1720	UACC1012	UACC1097	UACC903
TD1384	TD1730	TC1376-3				

As noted in the text, two pairs of specimens in Group A were derived from the same patient. The two pairs are M93-007 & M92-001 and TD1376-3 & TC1376-3. In our analyses, we only considered the data for each of these patients once or, as specifically noted, entirely removed the specimens for these patients from the analysis.

We first performed an analysis that included all specimen types (tissues and cell lines). We tested for associations between group and the following variables: sex, age, mutation status, biopsy site*, pigment, Breslow thickness, Clark level, and specimen type. There was no variable tested, which was shown to be associated with cluster group (at the 0.05 significance level.

Although there was not a statistically significant association between group and specimen type (p=0.106) it was noteworthy that all 5 tissue specimens were located in Group

* Biopsy site was broken down into the following three categories: skin/external (including ankle, abdomen/chest, shoulder, breast, neck/forehead and back), internal (including chest wall, distal ileum, paraspinous, thyroid lobe, small bowel, rectus muscle and intra-abdominal), and lymph nodes (including axillary, cervical and thigh femoral).

A. We therefore performed another analysis in which we only considered data from cell lines. In the analysis of cell lines, no variables were associated with cluster group at the 0.05 significance level, although "age" did have a marginal association (p=0.0812). Passage number was also tested in this analysis and had no association with group (p=0.8570).

Next, we investigated for differences in survival between the two cluster groups. We used a measure of survival that indicated survival time from the date of biopsy. Four cases (including the previous two) had a biopsy date falling in 1998 and a known status (alive or dead) for which a specific date of death or last follow-up was unknown. In order to use these cases in the survival analysis, the survival/follow-up time in these cases was arbitrarily set to 1 year if the biopsy date occurred prior to 7/1/98 or 0.5 years if the biopsy date occurred on or after 7/1/98.

The data used in the survival analysis are shown in Figure 1. A total of 15 cases were included in the analysis, 10 from Group A and 5 from Group B. Survival/follow-up times were rounded to the nearest quarter year. A Kaplan-Meier survival plot was created and log-rank test performed. No statistically significant association between group and survival was found (p=0.135).

The analyses performed resulted in no significant association with cluster group. However, this does not necessarily mean associations do not exist between the groups and the clinical and tumor characteristics tested. The power of the tests we performed is limited by the amount of data available for each variable. For example, only 6 specimens in Group A and 3 in Group B have information on Breslow thickness. Finding significant associations with so few data is unlikely. The power of the tests would increase with more complete data on the existing specimens and by the addition of new specimens to the data set. Such studies are underway in our laboratory.

ANALYSIS OF ALL SPECIMENS:

Group A = specimens that cluster; Group B = others.

Two pairs of specimens in Group A (M93-007/M92-001 & TD1376-3/TC1376-3) were derived from the same patient. The clinical and tumor characteristics for each of these patients are only considered once in the below analyses.

SEX - no statistically significant association with group

Contingency table with Fisher's exact test

	A	B	
F	4	4	p-value = 0.6754
M	12	7	alternative hypothesis: two-sided

AGE - no statistically significant association with group

Wilcoxon rank-sum test: p-value = 0.1397

data: x: age w/group = A , and y: age w/group = B

Mann-Whitney Statistic: W = 102.0, n=15, m=10

alternative hypothesis: two-sided

MUTATION STATUS - no statistically significant association with group

Contingency table with Fisher's exact test

	A	B	
mutated	2	4	p-value = 0.1713
deleted 6	1		alternative hypothesis: two-sided
WT	4	2	

Contingency table with Fisher's exact test

Combined mutated and deleted into one category.

	A	B	
mut./del.	8	5	p-value = 1
WT	4	2	alternative hypothesis: two-sided

BIOPSY SITE - no statistically significant association with group

Contingency table with Fisher's exact test

	A	B	
skin/external	3	3	p-value = 0.8763
internal	4	3	alt. hypothesis: two-sided
LN	7	4	

PIGMENT - no statistically significant association with group

Wilcoxon rank-sum test: p-value = 0.2631

Pigment Type: light=1, med=2, dark=3

(amelanotic = light; tan = med; pigmented = dark.)

data: x: pig. type w/group = A , and y: pig. type w/group = B

Mann-Whitney Statistic: W = 76.5, n=13, m=9

alternative hypothesis: two-sided

BRESLOW THICKNESS - no statistically significant association with group

Wilcoxon rank-sum test: p-value = 0.2619

data: x: thickness w/group = A , and y: thickness w/group = B

Mann-Whitney Statistic: W = 14.0, n=6, m=3

alternative hypothesis: two-sided

CLARK LEVEL - no statistically significant association with group

Wilcoxon rank-sum test: p-value = 0.4481

Clark level: II=2, III=3, IV=4

data: x: Clark level w/group = A , and y: Clark level w/group = B

Mann-Whitney Statistic: W = 19.5, n=6, m=5

alternative hypothesis: two-sided

For the below analysis, the two pairs of specimens in Group A derived from the same patient (M93-007/M92-001 & TD1376-3/TC1376-3) were removed.

5

SPECIMEN TYPE - no statistically significant association with group

Contingency table with Fisher's exact test

	A	B	
cell line	11	12	p-value = 0.106
tissue 4	0		alternative hypothesis: two-sided

ANALYSIS OF CELL CULTURES:

Group A = specimens that cluster; Group B = others.

A pair of cell lines in Group A (M93-007/M92-001) was derived from the same patient. The clinical and tumor characteristic for this patient is only considered once in the below analyses.

SEX - no statistically significant association with group

Contingency table with Fisher's exact test

	A	B	
F	4	4	p-value = 1
M	8	7	alternative hypothesis: two-sided

AGE - no statistically significant association with group

Wilcoxon rank-sum test: p-value = 0.0812

data: x: age w/group = A , and y: age w/group = B

Mann-Whitney Statistic: W = 80.0, n=11, m=10

alternative hypothesis: two-sided

MUTATION STATUS - no statistically significant association with group

Contingency table with Fisher's exact test

	A	B	
mutated	2	4	p-value = 0.1713
deleted	6	1	alternative hypothesis: two-sided
WT	4	2	

Contingency table with Fisher's exact test

Combined mutated and deleted into one category.

	A	B	
mut./del.	8	5	p-value = 1
WT	4	2	alternative hypothesis: two-sided

BIOPSY SITE - no statistically significant association with group

Contingency table with Fisher's exact test

	A	B	
skin/external	2	3	p-value = 0.7272
internal	2	3	alt. hypothesis: two-sided
LN	6	4	

PIGMENT - no statistically significant association with group

Wilcoxon rank-sum test: p-value = 0.4212

Pigment Type: light=1, med=2, dark=3

amelanotic = light; tan = med; pigmented = dark.

data: x: pig. type w/group = A , and y: pig. type w/group = B

Mann-Whitney Statistic: W = 50.5, n=9, m=9

alternative hypothesis: two-sided

BRESLOW THICKNESS - no statistically significant association with group

Wilcoxon rank-sum test: p-value = 0.2000

data: x: thickness w/group = A , and y: thickness w/group = B

Mann-Whitney Statistic: W = 8.0, n=3, m=3

alternative hypothesis: two-sided

CLARK LEVEL - no statistically significant association with group

Wilcoxon rank-sum test: p-value = 0.6349

Clark level: II=2, III=3, IV=4

data: x: Clark level w/group = A , and y: Clark level w/group = B

Mann-Whitney Statistic: W = 13.0, n=4, m=5

alternative hypothesis: two-sided

For the below analysis, the pair of specimens derived from the same patient in Group A (M93-007/M92-001) was removed.

PASSAGE NUMBER - no statistically significant association with group

Wilcoxon rank-sum test: p-value = 0.8570

Passage #'s for established cell lines were set equal to 21.

data: x: passage # w/group = A , and y: passage # w/group = B

Mann-Whitney Statistic: W = 34.0, n=8, m=8

alternative hypothesis: two-sided

Contingency table with Fisher's exact test

	A	B	
1-5	3	4	p-value = 0.8695
6-10	4	2	alternative hypothesis: two-sided
11-20	4	5	
>20	1	1	

SURVIVAL ANALYSIS:

Data used in the survival analysis:

Pt.ID	Group	Status	Time
M93-007	A	0	7
M91-054	A	0	7
UACC091	A	0	7
UACC502	A	1	0.5
UACC2534	A	1	0.25
TD1683	A	1	1
TD1720	A	0	0.5
TD1348	A	0	5
TD1730	A	0	0.5
TC1376-3	A	0	3
UACC827	B	1	0.5
UACC930	B	1	2.25
M93-047	B	0	6
TC-F027	B	1	1
UACC903	B	1	0.25

Status: 0 = alive, 1 = dead

Time is in years.

Example 2-Expression of *Wnt5a* in Cell Lines with Originally Low Level Expression

Wnt5a scored very high out of all the marker genes analyzed in the ability to discriminate between highly invasive malignant melanoma and less invasive melanoma. Melanoma samples with high levels of *Wnt5a* expression were more aggressive tumors than those with lower levels of *Wnt5a* expression. Figure 6 shows the top 22 genes selected for their ability to classify highly invasive malignant melanoma from less invasive melanoma. *Wnt5a* is at the tope of the list of these marker genes.

Figure 6 also shows *Wnt5a*'s expected signaling pathway in contrast to the *Wnt1* pathway. *Wnt1* is known to be transforming; however, its proximal methods of signaling are very difference from those of *Wnt5a*. In some studies, researchers have observed that the two pathways seem to oppose each other in terms of downstream effects. In the *Wnt5a* pathway, the first transduction of the *Wnt5a* signal is accomplished through the interaction of *Wnt5a* with a G protein-coupled receptor, frizzled 5 (FZD5). The signal is subsequently transduced through the PLC/IP3/DAG/PKC pathways. The *Wnt5a* signal eventually leads to integrin interactions, cytoskeletal effects, and other cellular effects.

Low level expression of *Wnt5a* in the cluster of 19 melanomas was verified by real time PCR. Data for the samples WM-1791C and UACC-1273 are shown in Figure 7. The real time PCR results show that there is much more *Wnt5a* transcript in cell line WM-1791C, which originally was scored as having high level expression of *Wnt5a* by gene chip analysis, than in UACC-1273, which was originally scored as having low level expression. Vectors used to express higher levels of Wnt5a in cells that normally express low levels were developed using standard techniques to see if the phenotype of less aggressive samples expressing low levels of Wnt5a could be changed. A derivative of UACC-1273, a transfectant 4-3, which had been transfected with this vector, shows an intermediate level of *Wnt5a* expression in the real time PCR analysis. The increase in *Wnt5a* expression carries over in WNT5A protein abundance as shown by Western blot and by immunohistochemical staining (nuclei staining blue, WNT5A staining red) (Figure 7).

In terms of morphology, cell lines with originally low levels of *Wnt5a* expression showed dramatic changes in morphology and cytoskeletal organization when stably transfected with a vector driving *Wnt5a* expression. The parental line, UACC-1273, is spindle shaped with few points of attachment to the culture plate and disorganized actin

filaments (Figure 8). The transfectants are broader and flatter with many extensions and highly polarized actin filaments.

In order to determine whether there was cross talk between the *Wnt5a* and *Wnt1* pathways, an assay looking at beta-catenin was used. When *Wnt1* signaling is active, beta-catenin is localized to the nucleus. In Figure 9, antibody staining for beta-catenin shows that the beta-catenin is localized in the cytoplasm and not concentrated in the nucleus. Therefore, no cross talk between the two pathways seems to be occurring.

Protein kinase C (PKC), a downstream target likely to be modulated by *Wnt5a*, was also looked at. *Wnt5a* modulates PKC activity by phosphorylation of some or all of the PKC isoforms and not by alteration of PKC transcript levels. As can be seen in Figure 9, increased phosphorylated PKC is produced in the transfectants expressing significant levels of the *Wnt5a* transcript, as expected. The isoforms must frequently phosphorylated are mu and alpha/beta. This is further evidence that one is looking at the exptected *Wnt5a* pathway. PKC is one of the central hubs of signal transduction, and pathways leading to many types of cellular action incuding proliferation, cytoskeletal organization, and cell movement are known.

Increased cell movement and invasiveness were also found to correlate with increased *Wnt5a* expression in a scratch assay and a Boyden chamber assay. Transfectants expressing increased levels of *Wnt5a* show increased competence in filling in open gaps on a cell culture dish when compared to cells of the parent cell line (Figure 10). Increased phosphorylated PKC was found to correlate with increasing cell invasiveness as measured by a standard test for invasiveness, the Boyden chamber assay.

The first transduction of the *Wnt5a* signal is accomplished through interaction with a G protein coupled, seven transmembrane receptor, frizzled 5. The various cell lines tested

show varying native levels of fzd5 transcript. In the cell line, UACC-1273, the transition from low to high *Wnt5a* expression is not associated with increasing amounts of the receptor. The use of an antibody to fzd5 prevents it from responding to *Wnt5a* and thereby attenuates or reverses the phenotypes that increased *Wnt5a* would normally produce. This is shown in the decreased level of phosphorylated PKC upon treatment with the anti-fzd antibody and in the decreased invasiveness of *Wnt5a* transfectants treated with the ant-fzd antibody.

Other Embodiments

The foregoing has been a description of certain non-limiting preferred embodiments of the invention. Those of ordinary skill in the art will appreciate that various changes and modifications to this description may be made without departing from the spirit or scope of the present invention, as defined in the following claims.